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# **Correlation Between Biomarkers of Exposure, Effect, and Potential Harm in the Urine of Electronic Cigarette Users**

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1 **Keywords: electronic cigarettes, cigarettes, non-smokers, metals,**  
2 **biomarkers**

3 **Abstract:**

4 **Objectives:** To determine if urinary biomarkers of effect and potential harm  
5 are elevated in electronic cigarette users compared to non-smokers and if  
6 elevation correlates with increased concentrations of metals in urine.

7 **Study Design and Setting:** This was a cross-sectional study of biomarkers  
8 of exposure, effect, and potential harm in urine from non-smokers (n=20),  
9 electronic cigarette users (n=20), and cigarette smokers (n=13).  
10 Participant's screening and urine collection were performed at the Roswell  
11 Park Comprehensive Cancer Center and biomarker analysis and metal  
12 analysis was performed at the University of California, Riverside.

13 **Results:** Metallothionein was significantly elevated in the electronic  
14 cigarette group ( $3761 \pm 3932$  pg/mg) compared to the non-smokers ( $1129 \pm$   
15  $1294$  pg/mg,  $p=0.05$ ). 8-OHdG (8-hydroxy-2' -deoxyguanosine) was  
16 significantly elevated in electronic cigarette users ( $442.8 \pm 300.7$  ng/mg) vs  
17 non-smokers ( $221.6 \pm 157.8$  ng/mg,  $p=0.01$ ). 8-isoprostane showed a  
18 significant increase in electronic cigarette users ( $750.8 \pm 433$  pg/mg) vs non-  
19 smokers ( $411.2 \pm 287.4$  pg/mg,  $p=0.03$ ). Linear regression analysis in the  
20 electronic cigarette group showed a significant correlation between cotinine  
21 and total metal concentration; total metal concentration and metallothionein;  
22 cotinine and oxidative DNA damage; and total metal concentration and

1 oxidative DNA damage. Zinc was significantly elevated in the electronic  
2 cigarette users ( $584.5 \pm 826.6 \mu\text{g/g}$ ) compared to non-smokers ( $413.6 \pm$   
3  $233.7 \mu\text{g/g}$ ,  $p=0.03$ ). Linear regression analysis showed a significant  
4 correlation between urinary zinc concentration and 8-OHdG in the electronic  
5 cigarette users.

6 **Conclusions:** This study is the first to investigate biomarkers of potential  
7 harm and effect in electronic cigarette users and to show a linkage to metal  
8 exposure. The biomarker levels in electronic cigarette users were similar to  
9 (and not lower than) cigarette smokers. In electronic cigarette users, there  
10 was a link to elevated total metal exposure and oxidative DNA damage.  
11 Specifically, our results demonstrate that zinc concentration was correlated  
12 to oxidative DNA damage.

13

#### 14 **What is the key question?**

- 15 • Is increased electronic cigarette usage associated with elevated metal  
16 exposure and if such exposure can cause biological harm?

#### 17 **What is the bottom line:**

- 18 • Biomarkers of exposure (cotinine and metals), effect (metallothionein),  
19 and potential harm (8-isoprostane and 8-OHdG) were elevated in  
20 electronic cigarette users and were similar to concentrations in  
21 cigarette smokers; also increased electronic cigarette usage (as

measured by cotinine) was correlated with elevated urinary metal concentrations, which were correlated with oxidative DNA damage.

### **Why read on:**

- This is one of the first studies to demonstrate a correlation between biological harm and electronic cigarette usage, suggesting the metal constituents (in particular zinc) in electronic cigarette aerosol can cause oxidative DNA damage. Given the recent deaths and pulmonary illnesses related to electronic cigarette usage, it is important for readers to know about the potential health effects related to electronic cigarette usage.

### **Strengths and Limitations:**

- This was a cross-sectional study with gender and age-matched populations to compare urinary biomarker levels and metal concentrations in electronic cigarette users versus cigarette smokers and non-smokers.
- This is the first study to demonstrate electronic cigarette users are exposed to increased concentrations of potentially harmful levels of metals (specifically zinc) that were correlated to elevated oxidative DNA damage.
- This study is based on a relatively small population (n=53) and small number of biomarkers and should be expanded.

- In the electronic cigarette and cigarette smoker groups, participants were not all using the same products and had different numbers of puffs/day.

## **Introduction:**

Cigarette smoking causes more than 480,000 deaths annually in the United States and is the leading cause of preventable death<sup>1</sup>. Electronic cigarettes, which grew in usage over 900% between 2011-2015, do not burn tobacco and may be a safer product<sup>2</sup>. However, there are limited scientific data to prove that electronic cigarettes are actually less harmful than combustible tobacco products, although they may be harmful in different ways. To the contrary, some previous research has demonstrated that electronic cigarette aerosols contain potentially harmful chemicals, such as acrolein; formaldehyde and benzene<sup>3</sup>; cytotoxic flavor chemicals, such as diacetyl and cinnamaldehyde<sup>4,5</sup>; metals and ultrafine particles including tin, chromium and nickel nanoparticles<sup>6,7</sup>; and free radicals<sup>8</sup>. Moreover, some electronic cigarette refill fluids and aerosols showed cytotoxicity when tested in vitro<sup>9,10</sup>, an effect that has been linked to metals in the refill-fluid<sup>6</sup>. An in vitro study demonstrated that isolated human alveolar macrophages exposed to electronic cigarette vapour induces inflammation and reduces phagocytosis leaving the patient more susceptible to pulmonary infections<sup>11</sup>. Moreover, recent case reports have attributed electronic cigarette use to several adverse health effects, such as respiratory diseases<sup>12</sup>, increased risk

1 for cardiovascular disease<sup>13</sup>, and impaired wound healing after surgery<sup>14</sup>.  
2 Several previous studies on electronic cigarettes have evaluated biomarkers  
3 of exposure in blood, urine, and saliva<sup>15,16,17</sup>, but none has yet examined and  
4 quantified biomarkers of effect and potential harm in relation to metals in  
5 electronic cigarette users.

6 This study compares urinary biomarkers of exposure, effect, and  
7 potential harm in non-smokers, conventional cigarette smokers, and  
8 electronic cigarette users and accounts for the effect of gender and age on  
9 biomarker expression. Based on the above studies, we hypothesized that  
10 there would be an increase in the level of biomarkers of effect and potential  
11 harm in electronic cigarette users compared to non-smokers and a decrease  
12 compared to cigarette smokers. The urinary biomarker of effect,  
13 metallothionein, is a protein that responds to and protects against metal  
14 toxicity and free radical stress. Urinary biomarkers of potential harm were  
15 two markers of oxidative stress: (1) 8-isoprostane, a prostaglandin formed by  
16 fatty acid peroxidation, and (2) 8-OHdG, a product of DNA oxidation. Urinary  
17 biomarkers of exposure were: (1) cotinine, a nicotine metabolite to measure  
18 smoking or vaping usage, and (2) total concentration of 11 urinary metals,  
19 which are present in electronic cigarette aerosol<sup>6,7,18</sup> and are known to  
20 associate with metallothionein<sup>19,20</sup>. Regression analyses were performed to  
21 identify relationships between biomarkers of exposure (cotinine and metals),  
22 effect (metallothionein), and potential harm (8-OHdG). To isolate the  
23 observed oxidative effects to a specific metal, regression analyses were

1 performed between the urinary concentrations of individual metals and 8-  
2 OHdG.

### 3 **Materials and Methods**

4       **Subjects:** The urine samples were from participants who were non-  
5 smokers, cigarette smokers, and electronic cigarette users. Participants were  
6 recruited through local media and flyers posted in various locations around  
7 the Buffalo, New York area. Potential participants were provided with a brief  
8 description of the study and had an opportunity to ask questions about the  
9 study procedures. All potential participants were screened over the phone for  
10 inclusion and exclusion criteria. The exclusion criteria included concurrent  
11 use of smokeless tobacco, pipes, or cigars; alcohol or illicit drug dependence  
12 within the past six months or current illicit drug use (including marijuana;  
13 self-reported); psychiatric illness; and use of Nicotine Replacement Therapy  
14 (NRT). Information about medication and vitamins/antioxidants/metal usage  
15 was not collected. All eligible subjects who had been asked to come to the  
16 clinic for screening were given an informed consent form to read and sign.  
17 Copies of the signed consent forms were given to the research subject and  
18 were also stored in a secure location, along with the participant's research  
19 chart. Informed written consent was obtained from each participant prior to  
20 their participation. Eligible participants were then asked to come to Roswell  
21 Park Comprehensive Cancer Center for a one-time visit, which lasted  
22 approximately 1 hour. Spot urine samples were collected during this on-site



1 visit. The Roswell Park IRB had reviewed all procedures prior to  
2 implementation (protocol number I 247313).

3 A total of 53 participants were gender and age matched and selected  
4 for biomarker analysis. Because age may affect the basal expression level of  
5 biomarkers, the subjects were separated into those  $\leq 40$  years old and  $\geq 41$   
6 years old, with the groups containing 23 and 30 samples, respectively. Out of  
7 these age-separated samples, participants were selected from the non-  
8 smoker, cigarette smoker, and electronic cigarette user groups. Each group  
9 had approximately equal male and female samples. Using a one-way ANOVA  
10 and a Tukey's multiple comparison test, there were no significant differences  
11 in the ages of the younger participants or in the ages of the older  
12 participants; however, the ages of the younger and older groups were  
13 significantly different from each other. There were negligible levels of 4-  
14 (Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in the non-smokers ( $2.8$   
15  $\pm 6.3$  pg/mg of creatinine) and electronic cigarette users ( $13.3 \pm 18.6$  pg/mg  
16 of creatinine) indicative of no tobacco use, in contrast to the cigarette  
17 smokers ( $105.7 \pm 87.4$  pg/mg of creatinine) who had significantly elevated  
18 NNAL (Supplementary Figure 1). In the non-smokers, no samples had levels  
19 of cotinine  $\geq 1.0$  ng/mg (Supplementary Figure 2), confirming smoking  
20 abstinence. The demographics of the 53 participants who provided urine  
21 samples were organized by age, gender, and smoking group (Table 1).

- 1 **Table 1. Demographics of the 53 participants included in this study**
- 2 **separated by smoking group, age, and gender. All smoking groups**
- 3 **were gender and age matched.**

**Age ( $\leq 40$   
years old)**

**Non-  
Smokers**

Sample ID	Sex	Age	Average
33B	Male	23	
07B	Male	25	
38B	Male	29	
21B	Male	37	
16B	Male	40	$30.8 \pm 7.4$
06B	Female	27	
09B	Female	32	
42B	Female	33	
45B	Female	33	
44B	Female	38	$32.6 \pm 3.9$

**Cigarette  
Users**

Sample ID	Sex	Age	Average
02A	Male	28	$28 \pm 0$
21A	Female	24	
17A	Female	33	$28.5 \pm 6.4$

**E-Cigarette  
Users**

Sample ID	Sex	Age	EC Type	Average
04C	Male	19	Tank	
35C	Male	28	Tank	
17C	Male	30	Tank	
41C	Male	34	Tank	
16C	Male	40	Tank	$30.2 \pm 7.8$
06C	Female	29	Tank	
23C	Female	32	Tank	
21C	Female	33	Tank	
28C	Female	39	Tank	
27C	Female	40	Tank	$34.6 \pm 4.7$

**Age ( $\geq 41$   
years old)**

**Non-  
Smokers**

Sample ID	Sex	Age	Average
13B	Male	42	
27B	Male	46	
26B	Male	58	
34B	Male	58	
43B	Male	66	$54 \pm 9.8$
41B	Female	41	
04B	Female	46	
28B	Female	52	
29B	Female	59	
35B	Female	61	$51.8 \pm 8.5$

**Cigarette  
Users**

Sample ID	Sex	Age	Average
08A	Male	41	
23A	Male	49	
03A	Male	65	
28A	Male	66	
13A	Male	75	$59.2 \pm 13.8$
14A	Female	46	
06A	Female	49	
18A	Female	57	
33A	Female	59	
36A	Female	69	$56 \pm 9.1$

**E-Cigarette  
Users**

Sample ID	Sex	Age	EC Type	Average
31C	Male	45	Tank	
37C	Male	47	Tank	
05C	Male	57	Tank	
32C	Male	60	Tank	
03C	Male	66	Cartomizer	$55 \pm 8.9$
08C	Female	44	Tank	
13C	Female	50	Tank	
09C	Female	55	Tank	
88C	Female	55	Tank	
12C	Female	62	Tank	$53.2 \pm 6.7$

4

- 5 **Biospecimen Collection:** Spot urine samples were collected from
- 6 participants in a previous study<sup>16</sup>, and cotinine, NNAL, and creatinine
- 7 concentrations were determined at the Center for Disease Control (CDC) and
- 8 the Roswell Park Comprehensive Cancer Center (RPCCC), respectively.

1 Aliquots of 45 ml of fresh urine samples were transferred to 50-ml Falcon  
2 tube then centrifuged and immediately frozen at -20°C and stored at the  
3 RPCCC laboratory. Prior to shipping, samples were thawed, and 1.5 ml  
4 aliquots were transferred to smaller tubes and shipped frozen to University  
5 of California, Riverside (UCR) for biomarker analysis. The biomarker study  
6 was approved under IRB protocol HS-12-023 from UCR.

7 ***Selection of Biomarkers:*** Biomarkers were selected by studying previous  
8 literature pertaining to urinary biomarkers in smokers<sup>21,22,23,24,25</sup>. The selection  
9 criteria for our panel of urinary biomarkers was based on our goal to analyze  
10 metal exposure and oxidative stress (Table 2). To evaluate exposure,  
11 cotinine and metals were measured in urine samples. Metallothionein, which  
12 increases when metal exposure is elevated, was used as a biomarker of  
13 effect. Conventional cigarettes and electronic cigarettes generate free  
14 radicals that cause cellular oxidative stress<sup>8,26,27</sup>. Therefore, oxidative  
15 damage was evaluated in the three study groups by measuring urinary 8-  
16 isoprostane (a biomarker of lipid peroxidation) and 8-OHdG (a biomarker of  
17 DNA oxidation). Cigarette smoke and electronic cigarette aerosols contain a  
18 mixture of metals<sup>6,7,28</sup> that could lead to an increased production of  
19 metallothionein (a metal exposure and ROS scavenging biomarker), which is  
20 a cysteine-rich protein that functions in metal binding<sup>25</sup>. All selected  
21 biomarkers described above have been shown to be specifically associated  
22 with clinically relevant outcomes and diseases (Table 2).

23

**Table 2. Clinical diseases associated with biomarkers measured in this study.**

<b>Biomarker Type</b>	<b>Associated Diseases</b>	<b>References</b>
<b>Exposure</b>		
Selenium	Nausea, vomiting, "garlic breath", nail loss, hair loss, cardiovascular disease, cardiac arrest, cancer,	MacFarquhar 2010, See 2006, Rayman 2012
Zinc	Nausea, vomiting, epigastric pain, fatigue, hypertension, hemotoxicity, bronchospasms, hepatotoxicity, neurotoxicity, cancer	Fosmire 1990, Nriagu 2007
<b>Effect</b>		
Metallothionein	Cancer, cardiomyopathy, oxidative stress, heavy metal toxicity	Eckschlager 2009, Zhou 2008, Ruttkay-Nedecky 2013, Klaassen 2009
<b>Potential Harm</b>		
8-OHdG	Cancer, cardiovascular disease, neurodegenerative diseases	Kroese 2014, Valavanidis 2009, Kim 2015
8-Isoprostane	Coronary artery disease, atherosclerosis, interstitial lung disease, non-small cell lung cancer, breast cancer	Vassalle 2004, Morrow 2005, Montuschi 1998, Stathopoulos 2014, Rossner Jr 2006

**Urinary Creatinine Concentrations:** Spot urine samples were used since biomarkers would not necessarily be stable in samples collected over 24 hours. Because spot urine samples were used, it was necessary to normalize the data to creatinine, which is relatively stable in concentration over time. Creatinine concentrations in urine were analyzed at the RPCCC clinical

laboratory in Buffalo. There were no significant differences in creatinine concentrations in relation to gender or age (Supplementary Figure 3).

#### ***Biomarker of Exposure (Cotinine, NNAL and Metal Concentration)***

**Analysis:** Cotinine and NNAL were measured using previously published<sup>29,30</sup> and fully validated methods. Eleven elements/metals (antimony, cadmium, copper, indium, lead, nickel, rubidium, selenium, silver, titanium, and zinc) in urine samples were measured by inductively coupled mass spectrometry (ICP-MS) and used to calculate total urinary metal concentration. The 11 metals were selected for analysis because they have all been identified in electronic cigarette aerosols and are known to associate with metallothionein. There was no significant elevation of the total 11 metals in the smoking groups, though it is slightly elevated in the electronic cigarette group (Supplementary Figure 4). Details of metal analysis are given in the Supplementary Information.

**Biomarkers of Effect and Potential Harm Analysis Using ELISA:** Each ELISA kit was quality tested for accuracy and reproducibility using urine samples collected in house. Samples were tested in duplicate on three different days, and the biomarker concentration was normalized to creatinine. A range of sample dilutions was tested to determine the optimal dilution for quantification of each biomarker from the kits' standard curves. For all ELISA kits, the coefficient of variation for the three independent

1 experiments was  $\leq 15\%$ , except for metallothionein, which was  $\leq 20\%$ . Any  
2 urine sample with a biomarker concentration outside the lowest or highest  
3 limit of quantification was excluded for statistical analysis. In all subsequent  
4 ELISA analyses, biomarkers were run in duplicate wells for each urine  
5 sample.

6       Following a 1:4 dilution in buffer, urine samples were analyzed to  
7 determine 8-isoprostane concentration using the Urinary 8-Isoprostane ELISA  
8 kit (Detroit R&D, MI, USA). The concentration of 8-OHdG was determined  
9 using a DNA Damage (8-OHdG) ELISA Kit (Stress Marq Biosciences, Victoria,  
10 Canada), following a 1:20 dilution. Urine samples were analyzed for  
11 metallothionein using a Human Metallothionein ELISA Kit (LifeSpan  
12 BioSciences, WA, USA), following a 1:20 or 1:40 dilution in sample diluent.

13

14 **Statistical Analysis:** Two urine samples from the electronic cigarette group  
15 had abnormally high creatinine concentrations ( $\geq 3$  mg/mL) as detected by a  
16 statistical outlier test and were removed from further analysis. For each  
17 urine sample, the biomarker concentration was normalized to its respective  
18 creatinine concentration. Because the normalized biomarker concentration  
19 data were not normally distributed, a Box-Cox transformation was performed  
20 after which a 3-way ANOVA was applied in MiniTab 17.0 (MiniTab Inc, PA,  
21 USA) using gender, age, and smoking group as factors. Outliers were  
22 removed if they had a large standardized residual ( $\geq 2.0$  or  $\leq -2.0$ ). In all the  
23 3-way ANOVA models, the 2-way and 3-way interactions were not significant,

and our final model included age, gender, and smoking group. Post-hoc tests were used to compare different age groups, gender groups, and smoking groups. When the smoking group was analyzed independently (disregarding gender and age), a Dunnett's post-hoc test was used with the electronic cigarette group as the main comparison group, and the comparisons were electronic cigarette users vs. non-smokers and electronic cigarette users vs. cigarette smokers. All linear correlation analyses were performed using the Linear Regression Analysis ( $R^2$  and p-value reported) in PRISM 7.0 (GraphPad, CA, USA). All graphs reported in this manuscript were made in PRISM 7.0.

**Patient and Public Involvement:** No patients were involved in the research planning or design, nor were they involved in any aspect of the study besides urine collection. There are no plans to directly disseminate the results of the research to study participants. The dissemination of results will be achieved through publication or press release.

## Results

### **Biomarker of Effect**

Metallothionein, a biomarker of effect (due to metal and reactive oxygen species exposure), in the electronic cigarette group ( $3761 \pm 3932$  pg/mg) was significantly elevated when compared to the non-smokers group ( $1129 \pm 1294$  pg/mg,  $p=0.05$ ), and these concentrations were similar to the

cigarette smokers group ( $4096 \pm 4320$  pg/mg,  $p=0.95$ ) (Figure 1A). There were no differences in age or gender.

### ***Biomarkers of Potential Harm (Oxidative Stress)***

A significant elevation in urinary levels of the biomarker of DNA oxidation, 8-OHdG, occurred in electronic cigarette users ( $442.8 \pm 300.7$  ng/mg) vs. non-smokers ( $221.6 \pm 157.8$  ng/mg,  $p=0.01$ ) (Figure 1B). There was no significant difference between electronic cigarette users ( $442.8 \pm 300.7$  ng/mg) and cigarette smokers ( $388 \pm 235$  ng/mg,  $p=0.75$ ). Age affected 8-OHdG levels; those  $\geq 41$  years old ( $413.4 \pm 256.4$  ng/mg) had significantly elevated 8-OHdG compared to those  $\leq 40$  years ( $241.2 \pm 214.1$  ng/mg,  $p=0.02$ ) (Figure 1C). There was no effect on gender.

The lipid peroxidation biomarker, 8-isoprostane, showed a significant increase in electronic cigarette users ( $750.8 \pm 433$  pg/mg) vs. non-smokers ( $411.2 \pm 287.4$  pg/mg,  $p=0.03$ ) (Figure 1D). There was no significant difference between electronic cigarette users ( $750.8 \pm 433$  pg/mg) and cigarette smokers ( $784.2 \pm 546.1$  pg/mg,  $p = 0.96$ ). Moreover, the  $\geq 41$ -year-old population ( $777.6 \pm 481.5$  pg/mg) was significantly elevated in 8-isoprostane compared to those  $\leq 40$  years ( $392.6 \pm 246.9$  pg/mg,  $p=0.002$ ) (Figure 1E). In addition, 8-isoprostane was significantly elevated in females ( $741.8 \pm 489.3$  pg/mg) vs. males ( $484.9 \pm 345$ ,  $p=0.04$ ) (Figure 1F).

### ***Biomarkers of Exposure are Correlated with Oxidative DNA Damage in E-Cigarette Users***



Results of linear regression analyses performed on the non-smokers, cigarette smokers, and electronic cigarette users are presented in Fig 2 for the following correlations: (1) cotinine and total metal concentration (Fig 2A-C), (2) total metal concentration and metallothionein (Fig 2D-F), (3) cotinine and 8-OHdG (Fig 2G-I), and (4) total metal concentration and 8-OHdG (Fig 2J-L). There were no significant correlations in the non-smokers (Fig 2A, D, G, and J). In the cigarette smokers group, only total metal concentration and 8-OHdG were significant (Fig 2K,  $p=0.0003$ ). In the electronic cigarette users group, all linear regression analyses were significant: cotinine and total metal concentration (Fig 2C,  $p=0.02$ ), total metal concentration and metallothionein (Fig 2F,  $p=0.04$ ), cotinine and 8-OHdG (Fig 2I,  $p = 0.02$ ), and total metal concentration and 8-OHdG (Fig 2L,  $p = 0.007$ ).

#### ***Selenium and Zinc were Elevated in Electronic Cigarette Users***

Two of the 11 metals that were analyzed were significantly elevated in the electronic cigarette group. Selenium concentrations (Fig 3A) were significantly elevated in the electronic cigarette users ( $54 \pm 20.6 \mu\text{g/g}$ ) compared to non-smokers ( $41.8 \pm 14.1 \mu\text{g/g}$ ,  $p=0.04$ ) and cigarette smokers ( $39.7 \pm 17.3 \mu\text{g/g}$ ,  $p=0.05$ ). Zinc concentrations (Fig 3B) were significantly elevated in electronic cigarette users ( $584.5 \pm 826.6 \mu\text{g/g}$ ) compared to non-smokers ( $413.6 \pm 233.7 \mu\text{g/g}$ ,  $p=0.03$ ). Zinc in the electronic cigarette users

1 was not significantly elevated when compared to cigarette smokers ( $470.7 \pm$   
2  $223.6 \mu\text{g/g}$ ,  $p=0.17$ ).

### 3 ***Zinc was Correlated with Oxidative DNA Damage in Electronic*** 4 ***Cigarette Users***

5 Regression analysis were performed to compare urinary concentrations  
6 of selenium and zinc to 8-OHdG in the non-smokers, cigarette smokers, or  
7 electronic cigarette users (Fig 4). There were no significant correlations for  
8 selenium versus 8-OHdG (Fig 4A-C). In the electronic cigarette users only,  
9 zinc was significantly correlated to 8-OHdG ( $p=0.0066$ ) (Fig 4F) In non-  
10 smokers and cigarette smokers, zinc was not correlated to 8-OHdG (Fig 4A,  
11 B).

12

### 13 **Discussion:**

14 Consistent with our hypothesis, our study shows for the first time that  
15 biomarkers of effect and potential harm were elevated in the urine of the  
16 electronic cigarette users compared to non-smokers. Moreover, in electronic  
17 cigarette users, the levels of biomarkers of effect and potential harm were  
18 positively correlated with biomarkers of exposure to nicotine and metals.  
19 Importantly, electronic cigarette participants in our study did not report  
20 using other tobacco products and were not dual users of electronic cigarettes  
21 and conventional cigarettes. Before entering our study, all electronic  
22 cigarette users who were previous cigarette smokers had abstained from

1 smoking cigarettes for a minimum of six months, and abstinence was  
2 confirmed by undetectable NNAL (Supplemental Fig 1). Previous literature  
3 has shown that abstinence from cigarette smoking was concurrently linked  
4 to a decrease in levels of 8-isoprostane and 8-OHdG, which returned to non-  
5 smokers levels<sup>31</sup>. Taken together, the above information supports the  
6 conclusion that the elevation of 8-isoprostane and 8-OHdG in urine was  
7 associated with electronic cigarette use specifically. Surprisingly, we did not  
8 find a significant reduction in biomarkers of effect and potential harm  
9 between electronic cigarette users and cigarette smokers. This observation  
10 may be explained by the fact that electronic and conventional cigarettes and  
11 their aerosols have anatomical, chemical, and particulate differences, which  
12 may contribute to physiological harm in separate ways.

13 Cigarette smoke and electronic cigarette aerosol contain a mixture of  
14 metals and free radicals<sup>6,7,8,28,32</sup> that could be contributing to the oxidative  
15 harm in our participants. The metals in electronic cigarette aerosols come  
16 mainly from the metal components in the atomizer and the e-fluid that is  
17 heated in the atomizer<sup>7,33</sup>. Metal concentration in urine was positively  
18 correlated with cotinine concentration, indicating that metals were elevated  
19 with increased aerosol exposure.

20 Metal increase in urine is further supported by the observed elevation  
21 in metallothionein, which acts as a heavy metal-binding protein and also  
22 protects cells from oxidative stress by scavenging ROS<sup>25</sup>. Metallothionein  
23 normally binds physiological metals, such as zinc and copper, but can also

1 bind xenobiotic heavy metals such as cadmium, silver and arsenic<sup>25,34</sup> that  
2 are present in cigarette smoke<sup>35</sup> and electronic cigarette aerosols<sup>7</sup>.  
3 Metallothionein can also associate with at least 20 different  
4 elements/metals<sup>19,20</sup>, and 11 of these have been found in cigarette smoke<sup>28,36</sup>  
5 or e-cigarette aerosol<sup>6,7,18</sup> and were present in the urine of our participants.  
6 The increase in metallothionein in the electronic cigarette user group was  
7 positively correlated with increasing metal concentration in their urine and  
8 was likely a response to metals inhaled by the electronic cigarette users. In  
9 cigarette smokers, metallothionein was not significantly correlated with  
10 increasing metal concentration, suggesting other factors such as ROS may  
11 be contributing to its activation. Also, cigarette smoke can have a different  
12 composition of metals than e-cigarette aerosol<sup>6,7,18,28,36</sup>, which were not  
13 selected for in our 11 metal analysis, and therefore the total metal  
14 concentration in smokers was not correlated to cotinine concentration.

15 Elevation of toxic metals can induce oxidative stress<sup>37,38</sup>. In the  
16 electronic cigarette group, there was a significant correlation between total  
17 metals and oxidative DNA damage. A similar correlation was observed for  
18 the cigarette smokers. Lipid oxidation was not significantly correlated with  
19 metal concentration in either the electronic cigarette or cigarette smokers  
20 groups. There are multiple isoprostanes and isoprostane metabolites formed  
21 in-vivo during oxidative conditions<sup>39</sup>, and we measured only 8-isoprostane,  
22 which may account for the lack of correlation between lipid oxidation and  
23 metal concentration. In contrast, during DNA oxidation the guanine residue is

1 highly oxidized compared to the other nucleic bases, leading to the  
2 formation of a single DNA oxidation product (8-OHdG), which makes  
3 correlation to oxidative stress straightforward.

4 Both zinc and selenium, which were significantly elevated in the  
5 electronic cigarette user group, are present in electronic cigarette aerosols,  
6 usually higher concentrations than most other elements<sup>6,7</sup>. However, only  
7 zinc concentration was correlated with oxidative DNA damage in the  
8 electronic cigarette group. While zinc is required for normal human health,  
9 its elevation above normal levels has been associated with oxidative stress<sup>40</sup>.  
10 Our data provide the first evidence that electronic cigarette usage increases  
11 the risk of zinc exposure, which in turn causes oxidative DNA damage in  
12 humans. Selenium is also a required trace element that can cause harm  
13 when elevated<sup>41</sup>. While its elevation in electronic cigarette users was not  
14 linked to increased oxidative stress, future work may find that it has other  
15 adverse health effects.

16 Oxidative damage can lead to gradual harm of all organ systems<sup>42</sup> and  
17 if left unchecked can culminate in diseases such as atherosclerosis, coronary  
18 heart disease, pulmonary fibrosis, acute lymphoblastic leukemia, and lung  
19 cancer<sup>43</sup>. Of particular concern, increases in both 8-isoprostane and 8-OHdG  
20 were significantly greater in the older populations, suggesting that  
21 conventional cigarette users who give up smoking and switched to electronic  
22 cigarettes may be at greater risk for oxidative damage than young people  
23 who have not smoked previously. In the case of 8-isoprostane, females were

more affected than males, suggesting that women should not be encouraged by physicians to use electronic cigarettes, especially when pregnant. There were no significant differences in the elevated concentrations of oxidative harm biomarkers between electronic cigarette users and cigarette smokers, suggesting their organ systems are exposed to similar levels of oxidative damage.

## **Conclusions:**

Our data show for the first time that electronic cigarette use, which correlates with metal intake, leads to an elevation in metallothionein in the urine. The usage of e-cigarettes causes an increase in oxidative stress as measured by 8-OHdG and 8-isoprostane. E-cigarette users were exposed to elevated levels of selenium and zinc. The intake of metals (specifically zinc) is further correlated with increased oxidative damage to DNA. These data indicate that electronic cigarette use is not harm free and that prolonged use with elevation of oxidative stress may lead to disease progression. Given these observations, physicians should use caution in recommending the use of electronic cigarettes to their patients and should be alert to possible adverse health outcomes associated with electronic cigarette use. The biomarkers used in this study may be valuable in clinical practice when evaluating the health of electronic cigarette users.

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## Footnotes:

**Contributors:** SSC, CH, MG, PT were responsible for the study concept and design. SSC, MW and ANR performed the experiments for data collection. MG collected and shipped the urine samples to our lab. JL acted as a statistician. SMB helped design the use of the ICP-MS in TWL's lab and the analysis of the metal data. SSC, MW, CH, JL, MG, PT drafted the manuscript, and all authors read and provided comments on the manuscript. SSC, MW, and PT reviewed the data and take responsibility for the integrity and accuracy of the data. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted. SSC and PT are the guarantors.

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3

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6 Johnson & Johnson (as a member of the advisory board) outside the  
7 submitted work. Authors not named here have disclosed no conflicts of  
8 interest.

9

10 **Ethics Approval:** The study was approved by Roswell Park Comprehensive  
11 Cancer Center IRB (protocol number I 247313). The biomarker measurement  
12 study was approved under IRB protocol HS-12-023 from UCR.

13

14 **Data Sharing:** No additional data are available.

15

16 **Transparency:** The study guarantors affirm that this manuscript is an  
17 honest, accurate, and transparent account of the study being reported; that  
18 no important aspects of the study have been omitted; and that any  
19 discrepancies from the study as planned (and, if relevant, registered) have  
20 been explained.

21 **Figure Legends**

**Figure 1. Urinary metallothionein (pg/mg of creatinine), 8-OHdG (ng/mg of creatinine), 8-isoprostane (pg/mg of creatinine), are significantly elevated in e-cigarette users compared to non-smokers.** A. Metallothionein levels among the different smoking groups. B. 8-OHdG concentration in the different smoking groups. C. 8-OHdG concentration in the younger and older populations. D. 8-isoprostane levels among the different smoking groups. E. 8-isoprostane levels in the younger and older populations. F. 8-isoprostane levels in males and females. Bars are the means and standard deviations for each group. \*  $p = < 0.05$ ; \*\*  $p = < 0.01$ .

**Figure 2. Correlation between total metals and cotinine, metallothionein and total metals, 8-OHdG and cotinine, and 8-OHdG and total metals in urine.** A-C. Linear regression analysis comparing total metal ( $\mu\text{g/g}$  of creatinine) and cotinine concentration (ng/mg of creatinine) in urine of the non-smokers, cigarette smokers, and e-cigarette user groups. D-F. Linear regression analysis comparing metallothionein concentration (pg/mg of creatinine) and total metal concentration ( $\mu\text{g/g}$  of creatinine) in urine in the non-smokers, cigarette smokers, and e-cigarette users groups. G-I. Linear regression analysis comparing 8-OHdG (ng/mg of creatinine) and cotinine (ng/mg of creatinine) concentration in urine of the non-smokers, cigarette smokers, and electronic cigarette user groups. J-L. Linear

1 regression analysis comparing 8-OHdG (ng/mg of creatinine) and total metal  
2 ( $\mu\text{g/g}$  of creatinine) concentration in urine of the non-smokers, cigarette  
3 smokers, and electronic cigarette user groups. N/A = not applicable since  
4 levels of cotinine in non-smokers was negligible.

5 **Figure 3. Urinary selenium ( $\mu\text{g/g}$  of creatinine) and zinc ( $\mu\text{g/g}$  of**  
6 **creatinine) concentrations are significantly increased in the**  
7 **electronic cigarette users.** A. Selenium concentrations in the different  
8 smoking groups. B. Zinc concentrations in the different smoking groups. Bars  
9 are the means and standard deviations for each group. \*  $p = < 0.05$ .

11 **Figure 4. Zinc concentrations ( $\mu\text{g/g}$  of creatinine) are significantly**  
12 **correlated to oxidative DNA damage in the electronic cigarette**  
13 **users.** A-C. Linear regression analysis comparing selenium ( $\mu\text{g/g}$ ) of  
14 creatinine and 8-OHdG (ng/mg of creatinine) in urine of the non-smokers,  
15 cigarette smokers, and e-cigarette user groups. D-F. Linear regression  
16 analysis comparing zinc ( $\mu\text{g/g}$  of creatinine) and 8-OHdG (ng/mg of  
17 creatinine) in urine in the non-smokers, cigarette smokers, and e-cigarette  
18 users groups.

20 **Supplementary Figure 1. NNAL concentration (pg/mg of creatinine)**  
21 **among the different smoking groups.** Significant elevation of NNAL (a  
22 biomarker of tobacco exposure) was seen in the cigarette smokers. Bars are

the means and standard deviations for each group. \*  $p = < 0.05$ ; \*\*\*\*  $p = < 0.0001$ .

**Supplementary Figure 2. Cotinine concentration (ng/mg of creatinine) in the different smoking groups.** Cotinine concentration is elevated in the cigarette smokers and e-cigarette users compared to non-smokers. There is no difference between the cigarette smokers and e-cigarette users. Bars are the means and standard deviations for each group. \*\*  $p = < 0.01$ ; \*\*\*\*  $p = < 0.0001$ .

**Supplementary Figure 3. Urinary creatinine concentration (mg/mL) in different genders and age populations.** A. Creatinine concentrations in males and females. B. Creatinine concentrations in the younger and older population. There were no significant differences between genders or age groups. Bars are the means and standard deviations for each group.

**Supplementary Figure 4. The total concentration of 11 metals ( $\mu\text{g/g}$  of creatinine) in each smoking group.** There were no significant differences in the total metals concentrations in any of the smoking groups. Bars are the means and standard deviations for each group.